

## STEREOSELECTIVITY IN POLYPHENOL BIOSYNTHESIS

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### ABSTRACT

Stereoselectivity plays an important role in the late stages of phenylpropanoid metabolism, affording lignins, lignans, and neolignans. Stereoselectivity is manifested during monolignol (glucoside) synthesis, e.g., where the geometry (*E* or *Z*) of the pendant double bond affects the specificity of UDPG:coniferyl alcohol glucosyltransferases in different species. Such findings are viewed to have important ramifications in monolignol transport and storage processes, with roles for both *E*- and *Z*-monolignols and their glucosides in lignin/lignan biosynthesis being envisaged. Stereoselectivity is also of great importance in enantioselective enzymatic processes affording optically active lignans. Thus, cell-free extracts from *Forsythia* species were demonstrated to synthesize the enantiomerically pure lignans, (-)-secoisolariciresinol, and (-)-pinoresinol, when NAD(P)H, H<sub>2</sub>O<sub>2</sub> and *E*-coniferyl alcohol were added. Progress toward elucidating the enzymatic steps involved in such highly stereoselective processes is discussed. Also described are preliminary studies aimed at developing methodologies to determine the subcellular location of late-stage phenylpropanoid metabolites (e.g., coniferyl alcohol) and key enzymes thereof, in intact tissue or cells. This knowledge is essential if questions regarding lignin and lignan tissue specificity and regulation of these processes are to be deciphered.

### INTRODUCTION

Phenylpropanoid metabolism in vascular plants provides major classes of phytochemicals of specialized function and structure.<sup>1</sup> These include monolignols, lignans

and neolignans, structural cell wall polymers (lignins and the aromatic portion of suberins), flavonoids and condensed tannins (i.e., their phenylalanine-derived portion), and miscellaneous aromatics derived from cinnamic and *p*-hydroxycinnamic acids.

This chapter addresses the biosynthesis and function of *E*- and *Z*-monolignols, their glucosides, and the closely related lignans and neolignans; a third section discusses our current knowledge of subcellular compartmentalization of enzymes and metabolites involved in lignin and lignan biosynthesis. Note that lignin biosynthesis has been comprehensively described recently, and the reader is referred to these texts.<sup>2-6</sup> Flavonoid and condensed tannin biosynthesis is covered both in this book (see chapter by Hrazdina) and elsewhere.<sup>7-10</sup>

A particular goal of this chapter is to make the reader cognizant of the intriguing stereoselective transformations that occur within the latter stages of the phenylpropanoid pathway (i.e., those associated with the lignin, lignan, and neolignan branches). Each of the three subject areas is discussed in turn; much of the work described is drawn from studies conducted in the authors' laboratory.

### STEREOSELECTIVE TRANSFORMATIONS IN *E*- AND *Z*-MONOLIGNOL GLUCOSIDE BIOSYNTHESIS

Woody and herbaceous plants undergo cell wall reinforcement processes, in which primary wall expansion is followed by secondary wall thickening. During secondary thickening, lignins are deposited into the cell corners, middle lamella, and secondary wall. From the results of studies pioneered by Goring and coworkers,<sup>11,12</sup> it was concluded that the middle lamella and cell corners of woody xylem tissue mainly consist of lignins (together with small amounts of pectins). By contrast, the secondary wall layers are a composite matrix of cellulose, noncellulosic polysaccharides, and lignins.

It has long been proposed<sup>2</sup> that lignins from woody plants are exclusively derived from the three *E*-monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols (fig. 1) and that the ratio of each monolignol in lignins varies with species,<sup>2</sup> subcellular compartment,<sup>2</sup> and tissue type (e.g., normal versus reaction wood<sup>13</sup>). For example, in the gymnosperm *Picea mariana*, it appears that the *p*-coumaryl alcohol content of lignin is greatest in the cell corners and middle lamella, whereas that of coniferyl alcohol is greatest in the secondary wall.<sup>14</sup> Interestingly, the *p*-coumaryl alcohol content of reaction wood is also higher than its 'normal' wood counterpart as evidenced from a study of Douglas-fir (*Pseudotsuga menziesii*).<sup>15</sup>

Such findings suggest that the pattern of monolignol deposition during woody plant cell wall assembly is both temporally and spatially predetermined, and a number of investigations employing radiolabeled substrates and actively lignifying tissues have provided support to this view.<sup>16-18</sup> A similar situation is envisaged to hold for herbaceous plants and grasses.<sup>2</sup> How these biochemical events (i.e., initiation of lignification, deposition of specific monolignols at different stages of cell wall development) occur is essentially unknown. Consequently, the signal transduction mechanisms in developmental processes that regulate and control lignification need to be deciphered.

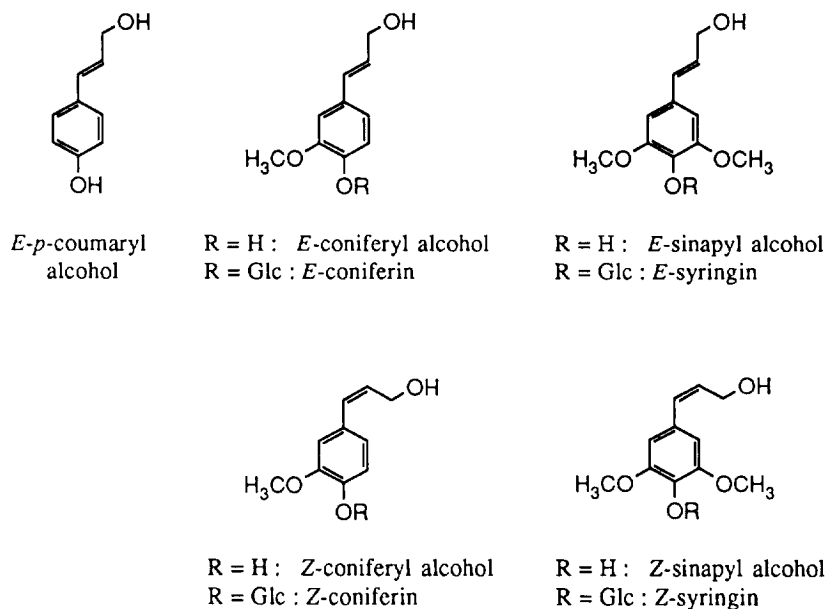


Figure 1.  $E$ - and  $Z$ -monolignols and their glucosides.

In addition to the three monolignols, hydroxycinnamic acids are also (apparently) introduced into the lignin polymer in herbaceous plants and grasses.<sup>19,20</sup> It is perhaps significant that these acids are often found as cell wall esters bound to noncellulosic polyoses, e.g., *O*-[5-*O*-*trans*-feruloyl- $\alpha$ -L-arabinofuranosyl]-(1  $\rightarrow$  3)- $\beta$ -D-xylanopyranosyl-(1  $\rightarrow$  4)-D-xylopyranose (FAXX).<sup>21</sup> We have proposed that such cell wall bound esters may serve as loci or recognition sites in the cell wall for lignification to be initiated.<sup>21</sup> Interestingly, cell wall bound esters (i.e., ferulate, *p*-coumarate) are found in both  $E$ - and  $Z$ -configurations in plants exposed to light, whereas in etiolated seedlings (e.g., of barley), only the  $E$ -isomer is detected.<sup>22</sup> In the absence of any contradictory evidence, it can be concluded that  $Z$ -hydroxycinnamate ester formation occurs via photochemical isomerization rather than by enzymatic control of stereochemistry. Supporting evidence for this notion comes from the facile light-induced isomerization of hydroxycinnamic acids *in vitro* to give  $E$ : $Z$  mixtures closely resembling those found in nature in living plants.<sup>21</sup>

Two distinct mechanisms have been proposed for monolignol transport from the cytoplasm through the plasma membrane and into the cell wall where lignification occurs.<sup>2,21</sup> In the first scenario, it is envisaged that monolignols are converted into their glucosides (e.g.,  $E$ -coniferin,  $E$ -syringin, fig. 1) and then cross through the plasma membrane. Action of a  $\beta$ -glucosidase regenerates the monolignols, and lignification occurs in a reaction catalyzed by peroxidase in the presence of  $H_2O_2$ . Alternatively, the monolignol glucosides may serve as storage products (e.g., in the vacuole) and are only conscripted for lignification as needed. In this case, the monolignols serve as the major species being transferred into the cell wall. Whatever the case, monolignol glucosides are assumed to play a role in the lignification process.

Unfortunately, this depiction of *E*-monolignol and *E*-monolignol glucoside formation, transport, and storage is overly simplistic. It provides no explanation for (a) the accumulation of *Z*-monolignols<sup>2,23,24</sup> and their glucosides<sup>2,23-25</sup> (but not their *E*-counterparts) in *Fagus grandifolia*, (b) the selective deployment of monolignols, such as coniferyl alcohol, as precursors for lignan<sup>26-28</sup> and neolignan biosynthesis, and perhaps for suberization, and (c) the spatial and temporal deposition of specific monolignols into the lignin polymer at different stages of cell wall maturation.<sup>2</sup> The resolution of such questions is an important goal of this laboratory.

Examination of *Fagus grandifolia* bark tissue revealed that only the *Z*-monolignols, *Z*-coniferyl and *Z*-sinapyl alcohols, and the *Z*-monolignol glucosides, *Z*-coniferin, *Z*-syringin (fig. 1), and *Z*-isoconiferin accumulated; the *E*-isomers were not detected.<sup>23,25</sup> This suggested that an alternate biosynthetic pathway to *Z*-monolignols was occurring in this species [perhaps involving the corresponding *Z*-hydroxycinnamic acids], as well as implying a different stereochemical basis for lignification; i.e., using *Z* rather than *E*-monolignols. The first stereochemical question was resolved<sup>29</sup> by a series of radiotracer experiments using [U-<sup>14</sup>C]Phe and [8-<sup>14</sup>C] *E*- and *Z*-ferulic acids, where it was established that [U-<sup>14</sup>C]Phe and [8-<sup>14</sup>C] *E*-ferulic acid served as precursors of *Z*-coniferyl alcohol, while [8-<sup>14</sup>C] *Z*-ferulic acid did not. Next, when [8-<sup>14</sup>C] *E*-coniferyl alcohol was incubated with *F. grandifolia* bark tissue, a significant conversion into the *Z*-isomer (*Z*-coniferyl alcohol) occurred. It must be stressed that, while photochemical isomerization of *E*- and *Z*-monolignols and their glucosides is attainable in vitro using an open face mercury arc lamp,<sup>30</sup> the biochemical conversions were obtained under conditions where photoisomerization was not detectable.<sup>29</sup> Thus, our results suggest the involvement of a novel stereoselective *E*→*Z* hydroxycinnamyl alcohol isomerase.

Given the presumed role(s) of monolignol glucosides in lignification, it was of considerable interest to next establish whether UDPG:coniferyl alcohol (CA) glucosyl-transferases exhibited any substrate stereoselectivity toward either *E*- or *Z*-monolignols.

Thus, cell-free extracts prepared from the angiosperm, *Fagus grandifolia*, were incubated with either *E*- or *Z*-coniferyl alcohol in the presence of UDP- $\alpha$ -D-[U-<sup>14</sup>C] glucose.<sup>31</sup> After incubation, both *E*- and *Z*-coniferins were added to the enzyme assay mixtures as radiochemical carriers. *E*- and *Z*-coniferins from each incubation were then separated by high-performance liquid chromatography, individually acetylated, and recrystallized to constant radioactivity. The results are given in table 1 and reveal a marked stereoselective preference for *Z*-coniferyl alcohol over its *E*-counterpart (5.74 percent versus <0.24 percent conversion, respectively).

In an analogous manner, a crude cell-free extract from the gymnosperm, *Pinus taeda* (loblolly pine), was prepared, and the stereoselectivity of the glucosylation reaction in coniferin synthesis was investigated. In this case, the glucosyltransferase preparation displayed only a slight preference for the *E* (over the *Z*) isomer, and both *E*- and *Z*-monolignols were efficiently glucosylated (see table 2).<sup>32</sup>

These findings reveal intriguing nuances in the stereoselectivity of UDPG:coniferyl alcohol glucosyltransferases that were previously unknown; i.e., UDPG:CA

Table 1. Stereoselectivity of *F. grandifolia* Bark UDPG:CA Glucosyltransferase

Monolignol substrate	Radiochemical carrier	Radiochemical conversion (percent)
<i>Z</i> -coniferyl alcohol	<i>E</i> -coniferin	0.0
	<i>Z</i> -coniferin	5.74
<i>E</i> -coniferyl alcohol	<i>E</i> -coniferin	<0.24
	<i>Z</i> -coniferin	0.92

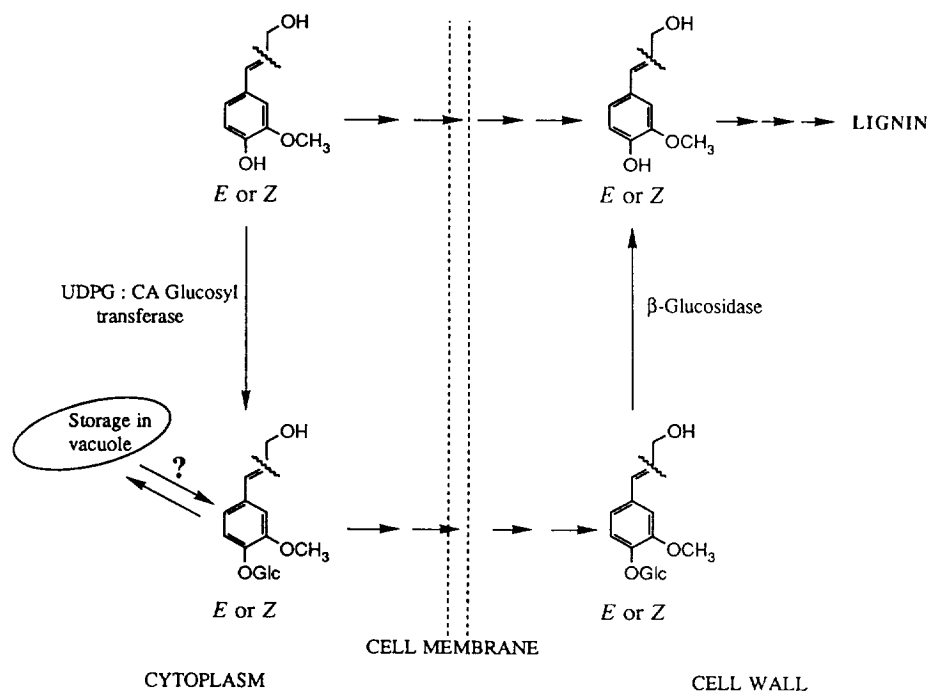
glucosyltransferases from various sources display profoundly different specificities toward *E*- and *Z*-monolignols. As a consequence, a revised depiction of monolignol transport and storage using either *E*- or *Z*-monolignols is proposed in figure 2. These results raise a number of new questions: (a) How do glucosyltransferases from different species effect such differences in stereoselectivity?, (b) Are distinct glucosyltransferases (i.e., isozymes) formed at different stages of cell wall development, and do they have differing specificities for *E*- and *Z*-coniferyl and sinapyl alcohols? (c) How efficiently are *E*- and *Z*-monolignol glucosides transported through the plasma membrane?, (d) Do the  $\beta$ -glucosidase(s) in the cell wall display different specificities for *E*- and *Z*-monolignol glucosides?, and (e) Where are the glucosyltransferases and monolignols located in the cell? Answers to such questions will clarify many of the uncertainties surrounding monolignol transport and storage and, consequently, the lignification process itself.

### STEREOSELECTIVITY IN LIGNAN BIOSYNTHESIS

Neolignans and lignans constitute an important and structurally diverse array of abundant phytochemicals. They are widely distributed in dryland angiosperms and gymnosperms (ranging from woody trees to constituents of vegetable fiber and grain) and have numerous physiological and pharmacological roles.<sup>1</sup> For example,

Table 2. Stereoselectivity of *P. taeda* Cambial Tissue UDPG:CA Glucosyltransferase

Monolignol substrate	Radiochemical product	Radiochemical conversion (percent)
<i>Z</i> -coniferyl alcohol	<i>Z</i> -coniferin	27.54
<i>E</i> -coniferyl alcohol	<i>E</i> -coniferin	57.52



**Figure 2.** Hypothetical transport and storage functions of *E*- and *Z*-monolignol (glucosides), such as coniferyl alcohol, in lignification.

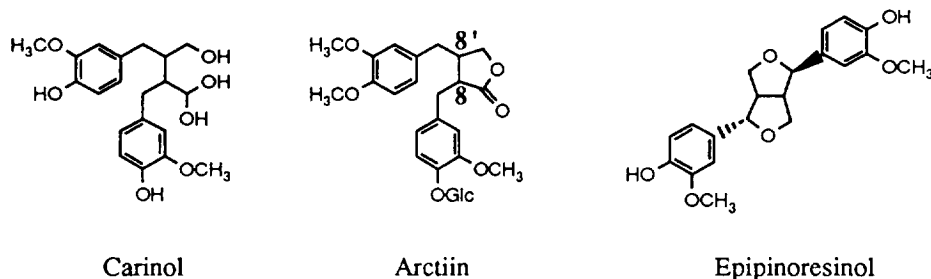
some are phytoalexins,<sup>33</sup> whereas others function in plant protection<sup>34–40</sup> against fungi, bacteria, insects, and other herbivores. They have also been implicated in lignin formation,<sup>41,42</sup> and many display important pharmacological properties in man, including anticancer activity.<sup>43–52</sup>

Lignans and neolignans are constructed through linkages between phenylpropanoid [ $C_6C_3$ ] units, linked either by 8–8' [ $\beta$ – $\beta'$ ] bonds to give lignans, or via alternate linkages affording the neolignans (for representative examples, see fig. 3).<sup>53</sup> By 1978, only 200 structural variants were reported,<sup>54</sup> but this number has grown enormously since. Lignans have been found in almost all plant types, and not just woody species as is sometimes erroneously assumed. As is to be expected, the relative amounts can vary substantially with plant species.

Lignans and neolignans are normally dimeric  $C_6C_3$  derivatives, although higher oligomeric forms continue to be found.<sup>53</sup> Lignans are generally present in plants as substituted dibenzylbutanes, dibenzylbutyrolactones, furans, furanofurans, aryltetrahydronaphthalenes, and (less frequently) as *O,O'* bridged biphenyls. Neolignans are frequently linked via 3,3', 8,3', and 8–*O*–4' bonds but can also occur as benzo-furans, dihydrobenzofurans, and other structural types.

Two different mechanisms have been proposed to account for phenylpropanoid ( $C_6C_3$ ) coupling leading to the dimeric lignan skeleta. These are oxidative<sup>55</sup> and

## Lignans :



## Neolignans :

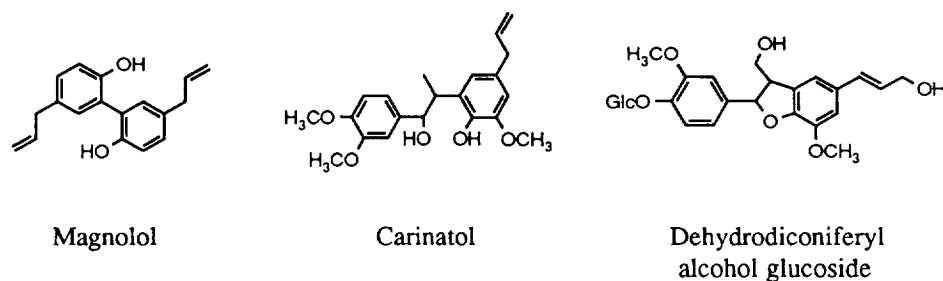


Figure 3. Representative examples of lignans and neolignans.

reductive<sup>56</sup> coupling reactions, with the former being most frequently cited; both mechanisms were based on dogma and not fact. This is not to imply that enzymatic coupling reactions had lacked experimental testing, and these studies can be traced back to early work in Freudenberg's laboratory,<sup>55</sup> where coniferyl alcohol was treated with horseradish peroxidase and  $\text{H}_2\text{O}_2$  in vitro. The products formed by this enzymatic coupling were mixtures of various compounds, including racemic ( $\pm$ )-pinoresinols (fig. 4). Herein lies the heart of the problem concerning lignan/neolignan biogenesis; naturally occurring lignans and neolignans are often not racemic, but most are found in an optically active form. Importantly, the sign of rotation of a particular lignan may vary with the plant species, e.g., in *Forsythia suspensa*, pinoresinol is reported to exist as its (+)-enantiomer,<sup>57</sup> whereas in *Xanthoxylum ailanthoides*, the (-)-antipode occurs.<sup>58</sup> Similarly, (+)-phillygenin occurs in *Forsythia intermedia*,<sup>59</sup> and the (-)-form is present in *Pararistolochia flosavis*.<sup>60</sup>

Until recently, there were few methods available to quickly and precisely establish the enantiomeric purity of a given lignan. This is not a trivial point, as illustrated by the wide range of  $[\alpha]_D$  and melting point values reported for syringaresinol (see table 3 and fig. 4) isolated from different plant sources,<sup>61</sup> and which suggest a large variation in enantiomeric composition between species.

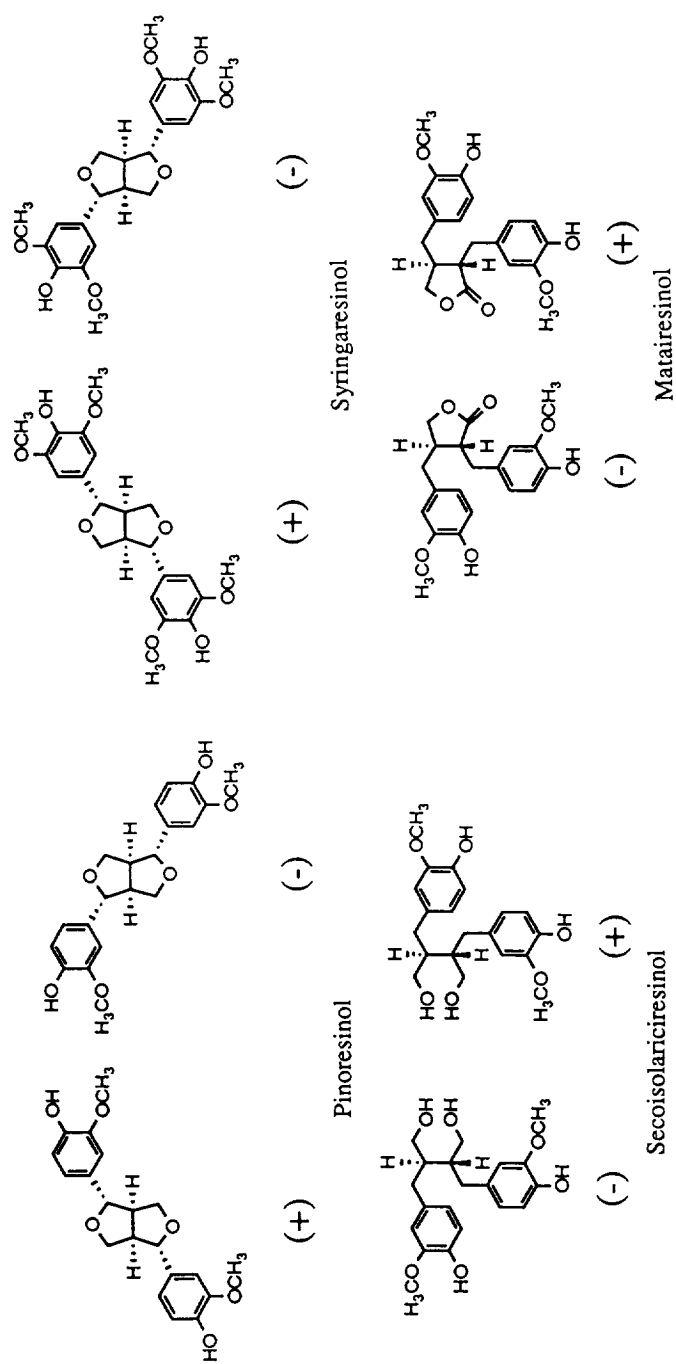


Figure 4. Enantiomeric forms of syringaresinol, pinoresinol, secoisolariciresinol and matairesinol.



**Table 3.** Specific Rotations and Melting Points of Syringaresinol Isolated from Different Species.<sup>61</sup>

Species	$[\alpha]_D$	Melting point (°C)
<i>Liriodendron tulipifera</i> <sup>62</sup>	+48.9	185–186
<i>Eucommia ulmoides</i> <sup>63</sup>	+44.0	183.5
<i>Hedyotis lawsoniae</i> <sup>64</sup>	+23.0	187–190
<i>Liriodendron tulipifera</i> <sup>65</sup>	+19.0	171–173
<i>Stellera chamaejasme</i> <sup>66</sup>	+3.0	n.d.
<i>Xanthoxylum inerme</i> <sup>67</sup>	0	179–185.5
<i>Daphne tangutica</i> <sup>68</sup>	- 2.1	174–176
<i>Xanthoxylum ailanthoides</i> <sup>58</sup>	- 9.6	175–180
<i>Holocantha emoryi</i> <sup>69</sup>	-32.5	184–187
<i>Aspidosperma marcgravianum</i> <sup>70</sup>	-34.8	177–183

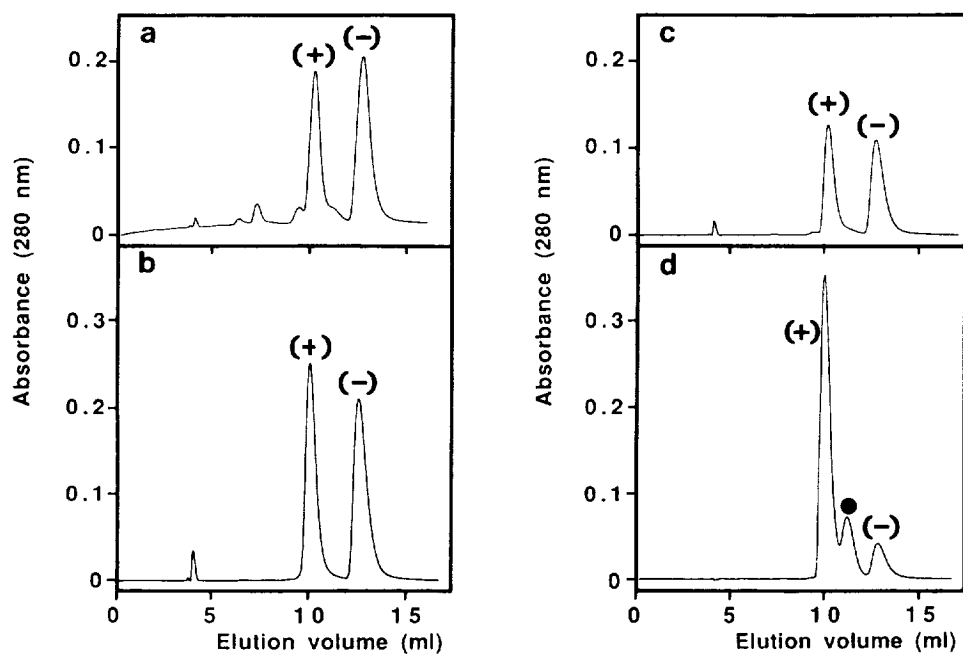
(n.d. = not determined)

Fortunately, such difficulties in determining enantiomeric purity of lignans have largely been overcome with the advent of chiral column high-performance liquid chromatography techniques.<sup>71</sup> Consequently, it is now possible to rapidly determine the optical purity of lignans from different plant sources. The results obtained for selected syringaresinol specimens are shown in figure 5 and table 4. As can be seen, this lignan exists in (+)-, (-)-, and (±)-forms, depending upon the plant species in question.

Such findings raise obvious questions regarding how stereoselective control is effected in phenylpropanoid coupling reactions. There appear to be only two ways to account for the optical activity of lignans: (1) Either the enzymatic coupling reaction is stereoselective and, therefore, cannot be a typical peroxidase-catalyzed reaction in the presence of H<sub>2</sub>O<sub>2</sub>, or (2) racemic products (or intermediates) are obtained, and one enantiomeric form is totally or partially converted into the other antipode or some other product.

Thus, to clarify the question about stereochemical control during phenylpropanoid coupling, we chose to probe the biosynthetic pathways to the *Forsythia* lignans, (+)-pinoresinol and (-)-matairesinol (fig. 4). Clearly, (+)-pinoresinol formation could occur via the coupling of two coniferyl alcohol moieties, but other possibilities (e.g., coupling of ferulic acid or coniferaldehyde moieties with subsequent reduction to afford pinoresinol) could not be discounted. In an analogous manner, (-)-matairesinol could be formed via direct coupling of one molecule of coniferyl alcohol with one molecule of ferulic acid, or via oxidation of an intermediate such as (-)-secoisolariciresinol (fig. 4), initially formed via the coupling of two coniferyl alcohol units.

To resolve whether stereochemical control occurs during phenylpropanoid coupling, three objectives needed to be met: (1) To determine whether pinoresinol,



**Figure 5.** Chiral separations of (+)- and (-)-syringaresinols isolated from different plant species: (a) *Xanthoxylum ailanthoides*,<sup>58</sup> (b) *Daphne tangutica*,<sup>68</sup> (c) *Xanthoxylum inerme*<sup>67</sup> and (d) *Eucommia ulmoides*,<sup>63</sup> • = impurity. HPLC conditions : Chiralcel OD column (4.6 x 250 mm, Daicel, Japan), eluted with EtOH, flow rate : 0.5 mL min.<sup>-1</sup>

**Table 4.** Comparison of Reported Optical Rotation  $[\alpha]_D$  Values with Enantiomeric Compositions of Syringaresinol Determined Following Chiral Column Chromatography and UV Detection

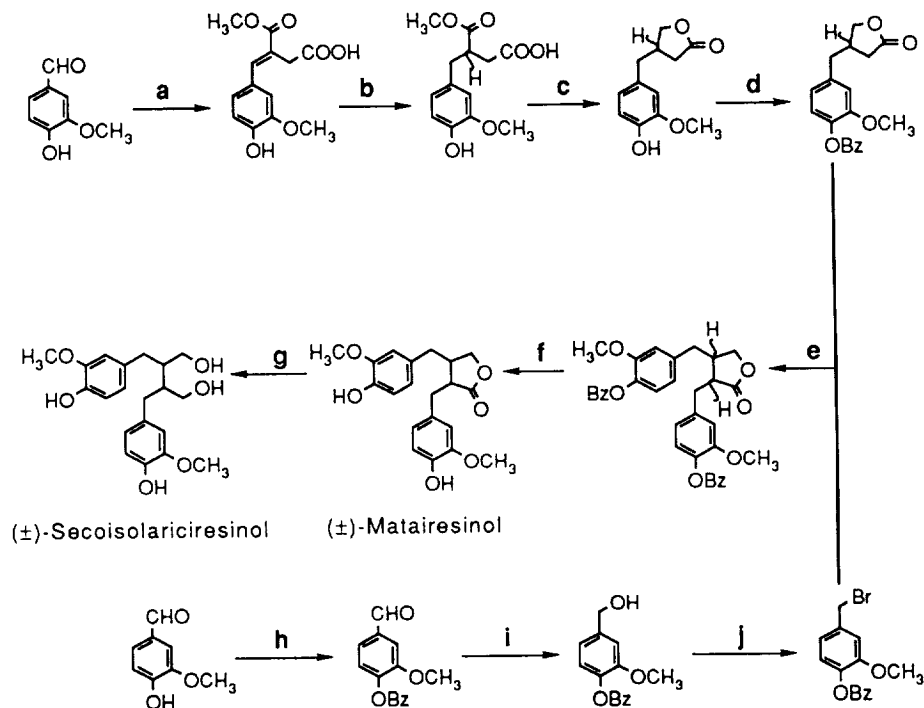
Species	$[\alpha]_D$	Syringaresinol (percent)	
		(+)	(-)
<i>Xanthoxylum ailanthoides</i> <sup>58</sup>	- 9.6	38	62
<i>Daphne tangutica</i> <sup>68</sup>	- 2.1	48	52
<i>Xanthoxylum inerme</i> <sup>67</sup>	0	48	52
<i>Eucommia ulmoides</i> <sup>63</sup>	+44.0	88	12

secoisolariciresinol, and matairesinol, are enantiomerically pure in *Forsythia* species, (2) to establish the chemical identity of the substrate(s) undergoing coupling and the immediate product(s) thereof, and (3) to determine whether the coupling reaction was enantioselective.

To determine the optical purity of each lignan in *F. suspensa* and *F. intermedia*, it was necessary to first obtain pinoresinol,<sup>26</sup> secoisolariciresinol,<sup>27,28</sup> and matairesinol<sup>27,28</sup> in racemic form by total synthesis (see scheme 1). [Note that a crucial step in the synthesis of (±)-matairesinols and (±)-secoisolariciresinols involved the *n*-BuLi/DIBAL-H reduction (step c) to give the lactone, which was otherwise difficult to achieve. This approach has since been taken by others in the synthesis of coniferyl alcohol-protein (BSA) conjugates.<sup>72</sup>]

Following the synthesis of the three racemic lignans, each was separated into its respective (+)- and (-)-forms by chiral column high-performance liquid chromatography (see fig. 6a-c). Subsequent analyses of the lignans revealed that only (+)-pinoresinol was present in *F. suspensa*, whereas in *F. intermedia*, only (-)-secoisolariciresinol and (-)-matairesinol occurred (fig. 7a-c); the corresponding antipodes were not detected.

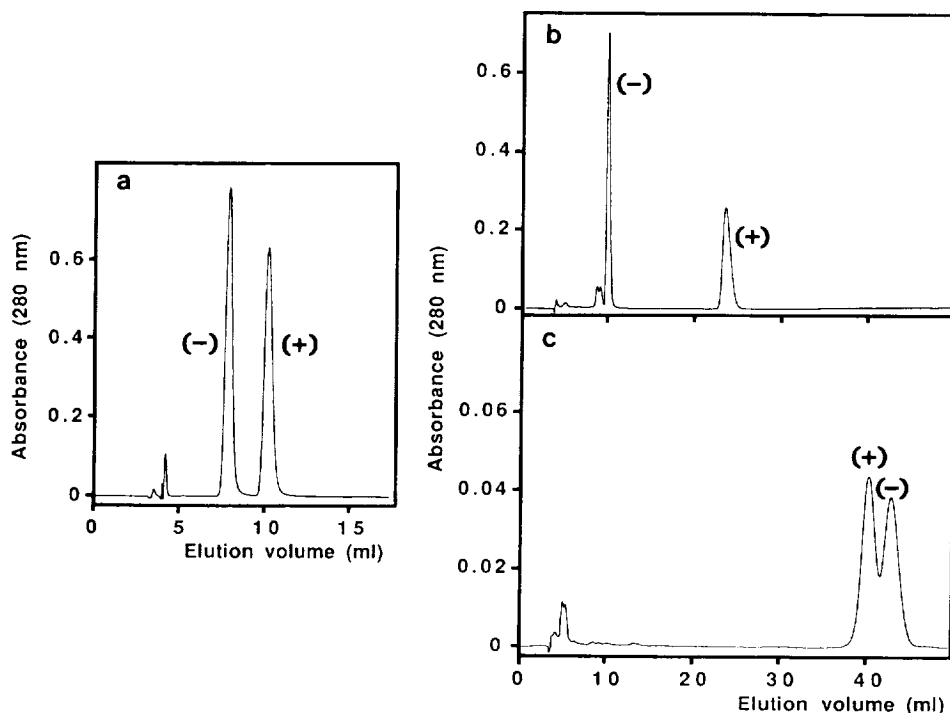
It was next determined that coniferyl alcohol served as the precursor of both (-)-secoisolariciresinol and (-)-matairesinol in *F. intermedia* tissue as follows:<sup>28</sup> [8-<sup>14</sup>C]coniferyl alcohol was administered to *F. intermedia* stem tissue, and following a 3-hour metabolism, the plant was homogenized with unlabeled (±)-secoisolariciresinols and (±)-matairesinols added as radiochemical carriers. Isolation and purification of each lignan revealed that radioactivity was coincident with elution of (-)-secoisolariciresinol and (-)-matairesinol and not with the (+)-antipodes. Experiments using cell-free extracts of *F. intermedia* stem tissue confirmed and extended these findings:<sup>27</sup> Incubation with [8-<sup>14</sup>C]coniferyl alcohol, in the presence of NAD(P)H and H<sub>2</sub>O<sub>2</sub>, and subsequent isolation of the enzymatically formed secoisolariciresinol revealed that only the (-)-form was radio-labeled. Final confirmation was afforded when [9,9-D<sub>2</sub>, OCD<sub>3</sub>]coniferyl alcohol, obtained as shown in scheme 2, was used as a substrate. The enzymatically formed (-)-deutero-secoisolariciresinol was isolated from the assay mixture preparation without addition of unlabeled product. Mass spectroscopic analysis of the resulting (-)-secoisolariciresinol gave a molecular ion *m/z*, 372 (*M*<sup>+</sup> + 10) and two fragments at *m/z* 354 (*M*<sup>+</sup> + 10 less H<sub>2</sub>O) and *m/z* 140 (benzylic cleavage with deuterated methyl group); i.e., it had been formed by the intact coupling of two [9,9-D<sub>2</sub>, OCD<sub>3</sub>]coniferyl alcohol molecules. Taken together, these results established the first example of stereoselective control during the biosynthesis of an optically active lignan. Similarly, experiments with *F. intermedia* (whole plants and cell-free extracts) demonstrated that the conversion of secoisolariciresinol into matairesinol was also stereoselective.<sup>28</sup> In this plant species, only the (-)-forms of [Ar-<sup>3</sup>H]- and [Ar-D]-secoisolariciresinol were converted into (-)-matairesinol; (+)-secoisolariciresinol was not a substrate for the formation of either (+)- or (-)-matairesinol. Thus, the stereoselective pathway to the *Forsythia* lignans, (-)-secoisolariciresinol and (-)-matairesinol, is as shown in scheme 3.



**Scheme 1.** Synthesis of (±)-matairesinols and (±)-secoisolariciresinols. Legend: (a) LiOMe, dimethylsuccinate, MeOH,  $\Delta$ , 43h (46 percent); (b)  $\text{H}_2$ , 10 percent Pd-C (92 percent); (c) DIBAL-H,  $n\text{BuLi}$  (49 percent); (d) benzylbromide,  $\text{K}_2\text{CO}_3$ , DMF (100 percent); (e)  $n\text{BuLi}$ /hexamethyldisilane (90 percent); (f)  $\text{H}_2$ , 10 percent Pd-C (97 percent); (g)  $\text{LiAlH}_4$  (92 percent); (h) benzyl bromide,  $\text{K}_2\text{CO}_3$ , DMF (71 percent); (i)  $\text{NaBH}_4$  (95 percent); (j)  $\text{PBr}_3$  (76 percent).

Pinoresinol exists exclusively as the (+)-antipode in both *F. intermedia* and *F. suspensa*. Consequently, when  $[\text{U-}^{14}\text{C}]$ phenylalanine was administered to *F. suspensa* stems,<sup>26</sup> radioactivity in the pinoresinol isolated after 3-hour metabolism was coincident only with the (+)-form, as expected. On the other hand, when  $[\text{8-}^{14}\text{C}]$ coniferyl alcohol was administered to *F. suspensa* stems or incubated with its cell-free extract in the presence of  $\text{H}_2\text{O}_2$ , (±)-pinoresinols were obtained, with the (+)-form slightly predominating.<sup>26</sup> However, when  $[\text{8-}^{14}\text{C}]$ coniferyl alcohol was incubated with *F. intermedia* cell-free extracts, but now in the presence of  $\text{NAD(P)H}$  and  $\text{H}_2\text{O}_2$ , as cofactors, only (−)-pinoresinol [and not the naturally occurring (+)-isomer] was formed. Note also that under these conditions, (−)-secoisolariciresinol is also obtained.

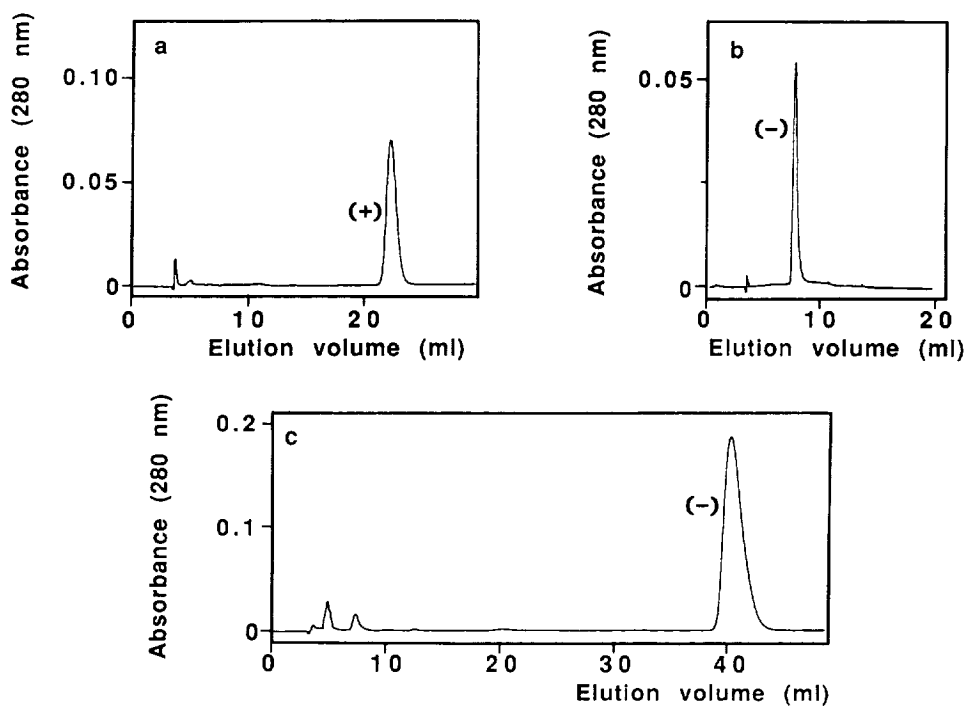
These results can be tentatively explained as follows: During uptake by the intact plant,  $[\text{U-}^{14}\text{C}]$ Phe is correctly compartmentalized, and the  $[\text{U-}^{14}\text{C}]$ coniferyl alcohol formed from it undergoes stereoselective coupling to give (+)-pinoresinol.



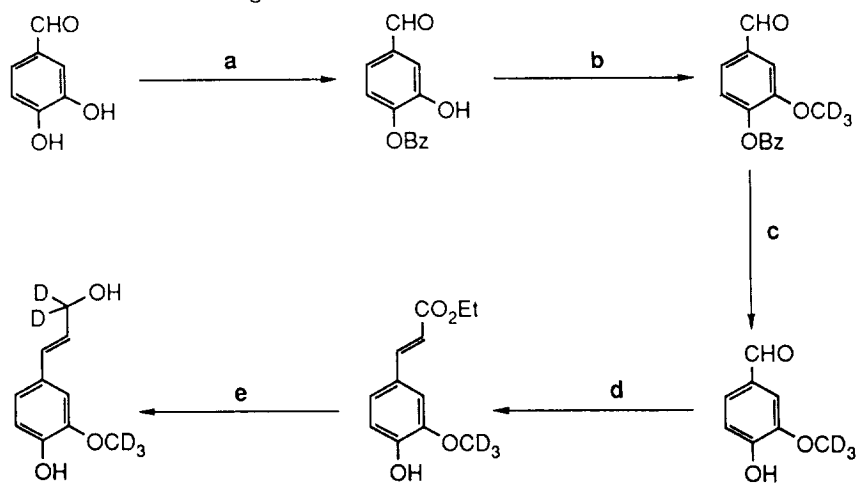
**Figure 6.** Chiral separation of racemic lignans: (a) pinoresinol, (b) secoisolariciresinol and (c) matairesinol. HPLC conditions : Chiralcel OD column (4.6 x 250 mm, Daicel, Japan); Solvent and flow rates: (a) pinoresinol: EtOH/hexanes 50:50, 0.8 mL min<sup>-1</sup>, (b) secoisolariciresinol: EtOH/hexanes 30:70, 0.5 mL min<sup>-1</sup> and (c) matairesinol: EtOH/1 percent AcOH in hexanes 15:85, 1 mL min.<sup>-1</sup>.

Conversely, when [8-<sup>14</sup>C]coniferyl alcohol was administered to intact plant tissue or cell-free extracts, it was not properly compartmentalized and was therefore subject to interference by nonspecific peroxidase-catalyzed coupling. Thus, as shown in figure 8, this interference by nonspecific peroxidases results in the formation of both (+)(8S,8S')- and (-)(8R,8R')-quinone methides as intermediates, which then undergo intramolecular cyclization to give (+)- and (-)-pinoresinols. This is not to imply, however, that racemic quinone methides are normally formed during lignan formation *in vivo* in *Forsythia* species. Under normal conditions *in vivo*, the metabolites would be properly compartmentalized, and stereoselective coupling would occur to afford the enantiomerically pure lignans.

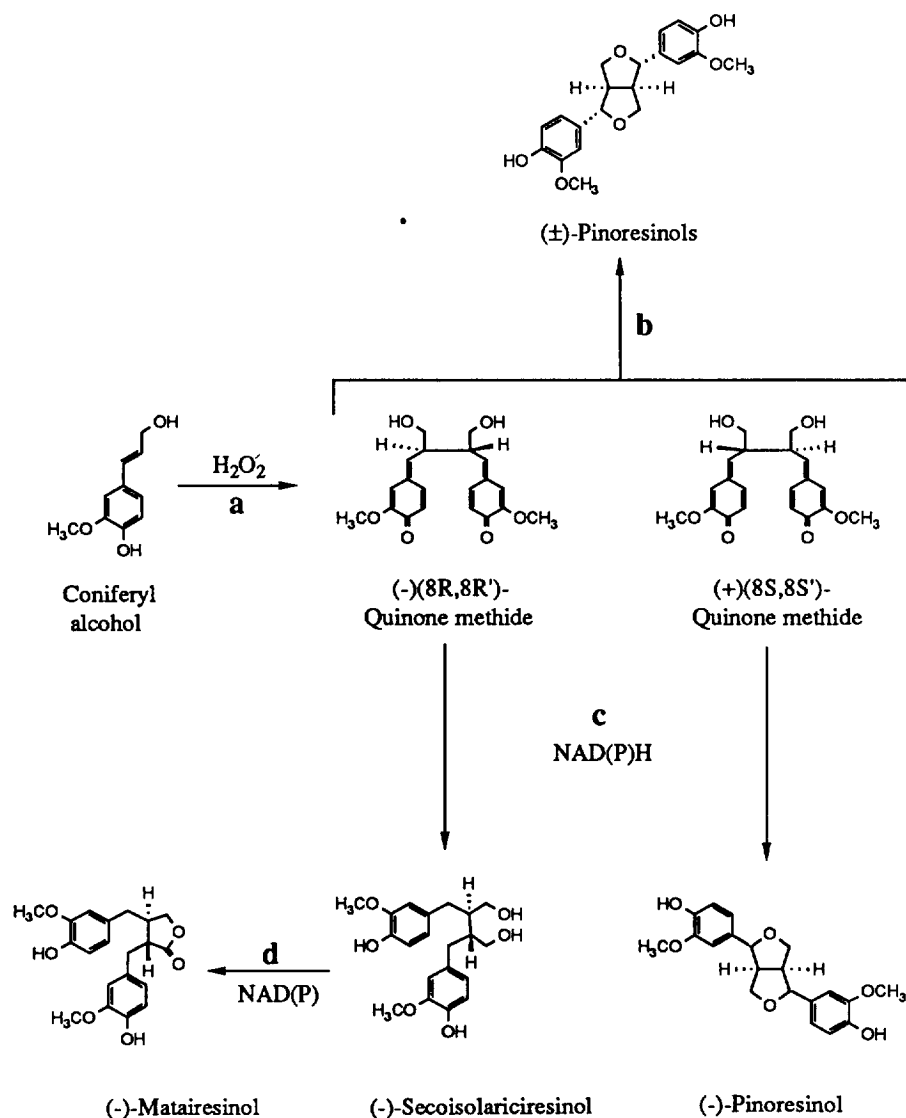
Unexpectedly, when NAD(P)H was added to the cell-free extract together with H<sub>2</sub>O<sub>2</sub>, racemic (±)-pinoresinols were not obtained. Instead the (-)-antipode was formed, which does not naturally occur in *F. intermedia* (see fig. 8, step c). This observation can be rationalized as follows: both (+)(8S,8S')- and (-)(8R,8R')-quinone methides are again formed *in vitro*, and only the (-)(8R,8R')-quinone methide



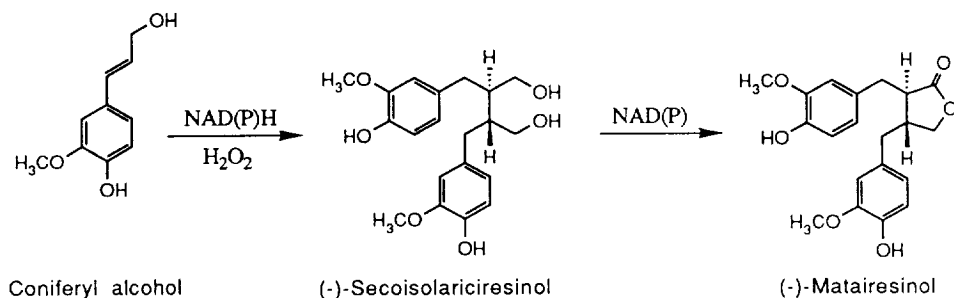
**Figure 7.** Chiral analysis of the lignans: (a) pinoresinol from *Forsythia suspensa* and (b) secoisolariciresinol, and (c) matairesinol from *Forsythia intermedia*. For HPLC conditions: see figure 6.



**Scheme 2.** Synthesis of [9,9-D<sub>2</sub>, OCD<sub>3</sub>]coniferyl alcohol. Legend: (a) benzyl bromide, K<sub>2</sub>CO<sub>3</sub> in DMF (32 percent); (b) CD<sub>3</sub>I (99 atom percent D), K<sub>2</sub>CO<sub>3</sub> in DMF (99 percent); (c) 30 percent wt HBr in AcOH, 90°C (79 percent); (d) monoethylmalonate, pyridine, aniline, piperidine, 52°C (82 percent); (e) LiAlD<sub>4</sub> (98 atom percent D) in Et<sub>2</sub>O (60 percent).



**Figure 8.** Proposed biosynthetic route to lignans, pinoresinol, secoisolariciresinol and matairesinol in *F. intermedia* cell-free extracts. Step: (a)  $\text{H}_2\text{O}_2$ /peroxidase coupling to give  $(\pm)(8R,8R';8S,8S')$ -quinone methides due to interference by non-specific peroxidases, (b) intramolecular cyclization of racemic quinone methides to give  $(\pm)$ -pinoresinols, (c) stereoselective reduction of  $(-)(8R,8R')$ -quinone methide to give  $(-)$ -secoisolariciresinol and intramolecular cyclization of  $(+)(8S,8S')$ -quinone methide to give  $(-)$ -pinoresinol and (d) stereoselective dehydrogenation to give  $(-)$ -matairesinol.



**Scheme 3.** Biosynthetic pathway to *Forsythia* lignans, (-)-secoisolariciresinol and (-)-matairesinol.

undergoes rapid stereoselective reduction to give (–)-secoisolariciresinol. By contrast, the (+)(8S,8S′)-quinone methide is not reduced and thus can only undergo intramolecular cyclization to give the unnatural antipode, (–)-pinoresinol.

Thus, the first demonstration of stereoselective enzyme-catalyzed transformations in lignan biosynthesis has been reported and offers the opportunity to address a number of fascinating questions: (1) What is the precise nature of the stereoselective coupling enzyme in *F. intermedia*? [This cannot be answered until the enzyme(s) has(have) been purified from competing nonspecific coupling enzymes]; (2) Are there two distinct coupling enzymes which are differently expressed or induced in different species? If so, this could explain the predominance of (+)-syringaresinol in *Eucommia ulmoides*,<sup>63</sup> and formation of (–)-syringaresinol in *Aspidosperma marcgravianum*.<sup>70</sup> Are both operative in other species?; (3) Are all post-coupling enzymatic transformations strictly stereoselective?; (4) Are lignan- and lignin-forming biochemical systems in different cells or are they separately compartmentalized, regulated, and controlled within the same cell?

Answers to such questions will clarify many of the outstanding mysteries remaining in the lignin, lignan, and neolignan branches of phenylpropanoid metabolism.

### COMPARTMENTALIZATION OF SPECIFIC PHENYLPROPANOID METABOLITES AND ENZYMES

The subcellular location(s) of late-stage phenylpropanoid metabolites (i.e., monolignols, monolignol glucosides, lignans) and the enzymes synthesizing such substances are unknown. Consequently, until this is rectified, much of our views on lignin and lignan formation can only be speculative. Determining where *E*- and *Z*-monolignol (glucosides) and specific lignans (e.g., secoisolariciresinol) accumulate, and where the enzymes synthesizing such metabolites are located, should provide an excellent start to deciphering synthesis, transport, storage, and regulatory processes. For example, it will be of particular interest to establish whether *E*- and *Z*-monolignols differ in subcellular location (i.e., bark vs. woody xylem tissue), whether monolignol glucosides are storage products (e.g., in the vacuole) or are actively transported through the plasma membrane, and whether lignan deposition processes are tissue-specific.



To achieve such goals, the precise subcellular location(s) of *E*- and *Z*-coniferyl alcohol and their glucosides, and the lignans secoisolariciresinol and matairesinol, need to be determined. Similarly, the site(s) of metabolite synthesis where the enzymes, cinnamyl alcohol dehydrogenase, UDP glucose:*E*- and *Z*-coniferyl alcohol glucosyltransferases and enzymes catalyzing the formation of secoisolariciresinol and matairesinol are located within the cell must be established. In this section, preliminary progress in establishing the location of *E*-coniferyl alcohol in intact tissue (or cells thereof) is described. Note that a parallel thrust is underway to determine the location of cinnamyl alcohol dehydrogenase involved in both lignan and lignin synthesis.

Before describing current progress toward achieving these goals, a brief summary of current technologies available to determine subcellular locations and their limitations is required. Until recently, the approaches commonly employed; i.e., cell fractionation, morphological analyses were of limited use. For example, cell fractionation techniques (i.e., isolation of chloroplasts, mitochondria, vacuoles, etc.) often did not give the desired resolution, where freedom from contamination from other subcellular constituents was assured. Consequently, results obtained were often open to subjective interpretation. Moreover, as far as light and electron microscopy are concerned, these techniques alone cannot be used to identify where a particular (macro)molecule or enzyme within a tissue cross-section is located.

Over the last decade or so, such difficulties have been substantially overcome by the application of different methods that permit the localization of specific molecules (e.g., abscisic acid,<sup>73</sup> partially methylated flavonoid glucosides<sup>74</sup>) and enzymes (e.g., ribulose-biphosphate carboxylase-oxygenase<sup>75</sup>) in intact tissues or cells. Such advances were made possible using immunocytochemistry; i.e., via colloidal gold labeling (= electron opaque markers) of an immunogenic response to an antibody, raised against either a (macro)molecule or protein. Visualization of the immunocytochemical response is obtained on a tissue or cell section using either electron or light microscopy. Although such immunocytochemical techniques are widely used, sensitive and specific, it must be emphasized that specificity is critically dependent upon the production of highly specific antibodies.

Turning our attention to determining the subcellular location of *E*-coniferyl alcohol in developing xylem tissue, it should first be realized that coniferyl alcohol is too small a molecule (mol. wt. 180) to generate an immunogenic response in mammalian systems (e.g., rabbits). It must first be conjugated with a larger immunogenic molecule or carrier protein, such as bovine serum albumin (BSA). Thus, the complex formed (see fig. 9) between *E*-coniferyl alcohol and BSA (i.e., the hapten:protein conjugate) was prepared by first reacting *E*-coniferyl alcohol with chloroacetic acid. The resulting acid was converted to its anhydride using dicyclohexylcarbodiimide (DCC) and subsequently covalently attached to BSA as the amide.

It should be noted that, typically, a hapten:protein ratio of 10:1 is necessary in order to obtain polyclonal antibodies of sufficient titer for immunocytochemical purposes. In our hands, an accurate determination of the coniferyl alcohol:BSA (hapten:protein) ratio could only be obtained using radiochemical techniques. Thus,

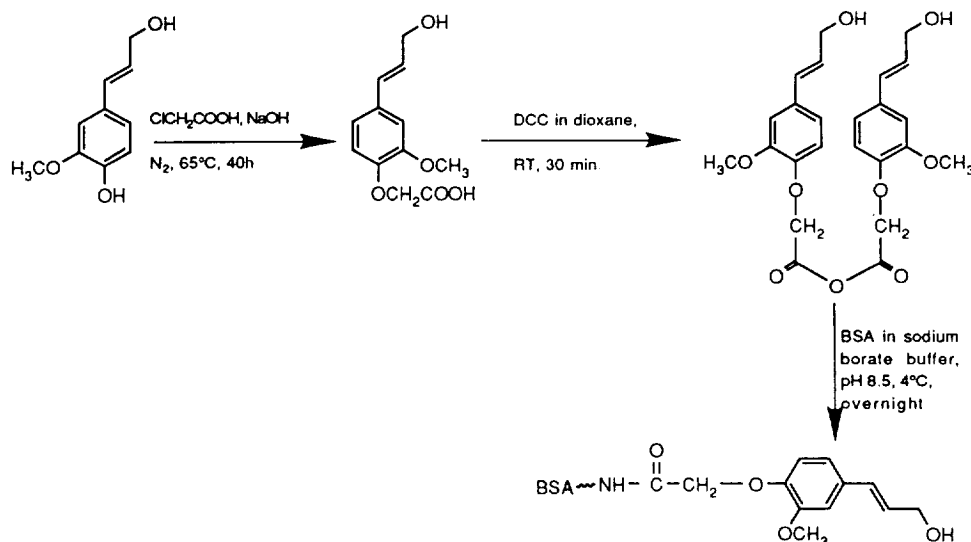


Figure 9. Synthesis of *E*-coniferyl alcohol : BSA conjugate.

coniferyl alcohol was reacted with  $[1-^{14}\text{C}]$ chloroacetic acid, with the resulting acid conjugated to BSA as described earlier, giving a hapten:protein ratio of 21.5:1. Note that UV estimations did not give accurate values of hapten:protein ratios.

The required *E*-coniferyl alcohol:BSA conjugate was applied intradermally across the back of the rabbit in a phosphate buffer saline (PBS) solution emulsified in Freund's complete adjuvant. This procedure was repeated three more times over a 1-week interval.

Two weeks after the last injection, additional conjugate was injected subcutaneously into the back of the neck. Booster injections were repeated every 2 weeks, and the serum was tested for antibody production by dot blots<sup>76</sup> a week after the injection. Antibodies were first detected in the serum 5 weeks following the first injection, but the conjugate was applied to the rabbit four more times, as before, in order to increase the titer of the polyclonal antibodies.

As alluded to earlier, immunogold labeling techniques are sensitive and specific, provided the antibody which is used as a probe is both specific and sensitive. Thus, prior to immunocytochemical localization of *E*-coniferyl alcohol in plant tissue, the specificity of the antibodies needed first to be examined using a number of compounds containing similar functionalities; i.e., to determine whether cross-reactivity was occurring. The selected compounds are given in table 5 and include phenylpropanoid metabolites such as *Z*-coniferyl alcohol, coniferaldehyde, ferulic acid, sinapyl alcohol, and coniferin; cross-reactivity was determined using the ELISA technique.<sup>76</sup>

As can be seen from table 5, antibodies raised against the *E*-coniferyl alcohol:BSA conjugate differentially cross-react with *Z*-coniferyl alcohol, coniferaldehyde, sinapyl alcohol, and coniferin (51 to 60 percent cross-reactivity) and to a lesser extent with ferulic acid (9 percent).

**Table 5.** Cross-reactivity of Anti *E*-Coniferyl Alcohol Serum<sup>a</sup>

Analogues	Cross-reactivity (percent)
<i>E</i> -coniferyl alcohol	100
<i>Z</i> -coniferyl alcohol	56
Coniferaldehyde	52
Ferulic acid	9
Sinapyl alcohol	51
Coniferin	60

<sup>a</sup>Cross-reactivity was determined by the enzyme-linked immunosorbent assay (ELISA) : The antiserum was preincubated with *E*-coniferyl alcohol or its structural analogues and further incubated with the antigen:protein conjugate immobilized on a solid phase.

It can, therefore, be anticipated that by chromatographic separation of the polyclonal antibodies, fractions will be obtained that will specifically react with *E*-coniferyl alcohol but not with the other analogues. A similar situation should hold also for *Z*-coniferyl alcohol, *E*- and *Z*-coniferins, and the corresponding lignans, secoisolariciresinol, pinoresinol, and matairesinol.

In summary, the preliminary results obtained indicate that it will be possible to produce antibodies highly specific to individual phenylpropanoid metabolites. A similar situation currently holds for cinnamyl alcohol dehydrogenase from *Pinus taeda*. Antibodies have been prepared and should permit establishing the subcellular location of this protein. Thus, a continued emphasis in this area should allow us to determine where synthesis, storage, and transport functions are located, as well as allowing us to distinguish between tissue-specific responses for lignin and lignan synthesis.

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